Adenoviral Expression of a Urokinase Receptor–Targeted Protease Inhibitor Inhibits Neointima Formation in Murine and Human Blood Vessels

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Background—Smooth muscle cell migration, in addition to proliferation, contributes to a large extent to the neointima formed in humans after balloon angioplasty or bypass surgery. Plasminogen activator/plasmin–mediated proteolysis is an important mediator of this smooth muscle cell migration. Here, we report the construction of a novel hybrid protein designed to inhibit the activity of cell surface–bound plasmin, which cannot be inhibited by its natural inhibitors, such as α_2-antiplasmin. This hybrid protein, consisting of the receptor-binding amino-terminal fragment of uPA (ATF), linked to the potent protease inhibitor bovine pancreas trypsin inhibitor (BPTI), can inhibit plasmin activity at the cell surface.

Methods and Results—The effect of adenovirus-mediated ATF.BPTI expression on neointima formation was tested in human saphenous vein organ cultures. Infection of human saphenous vein segments with Ad.CMV.ATF.BPTI (5×10^9 pfu/mL) resulted in 87.5±3.8% (mean±SEM, n=10) inhibition of neointima formation after 5 weeks, whereas Ad.CMV.ATF or Ad.CMV.BPTI virus had only minimal or no effect on neointima formation. The efficacy of ATF.BPTI in vivo was demonstrated in a murine model for neointima formation. Neointima formation in the femoral artery of mice, induced by placement of a polyethylene cuff, was strongly inhibited (93.9±2%) after infection with Ad.CMV.mATF.BPTI, a variant of ATF.BPTI able to bind specifically to murine uPA receptor; Ad.CMV.mATF and Ad.CMV.BPTI had no significant effect.

Conclusions—These data provide evidence that adenoviral transfer of a hybrid protein that binds selectively to the uPA receptor and inhibits plasmin activity directly on the cell surface is a powerful approach to inhibiting neointima formation and restenosis. (*Circulation.* 2001;103:562-569.)

Key Words: plasminogen ■ restenosis ■ gene therapy ■ urokinase ■ receptors

Smooth muscle cell (SMC) proliferation and migration play a major role in neointima formation and restenosis.[1,2] It has been suggested that the role of SMC migration is strongly underestimated and that SMC migration from media and adventitia to the (neo)intima is essential in neointima formation.[3,4]

Cell migration requires coordinated detachment and renewal of cell-matrix interaction sites, a process in which proteolytic enzymes are involved. Involvement of the plasminogen activation/plasmin system in the regulation of SMC migration in vitro and neointima formation in vivo has been demonstrated in several studies.[5–7] Inhibition of protease activity, either by knocking out specific proteases or by overexpressing protease inhibitors such as plasminogen activator inhibitor (PAI)-1 or tissue inhibitors of metalloproteinases (TIMPs), has resulted in inhibition of neointima formation, but the observed inhibition was either ≈50% or ≈79% for TIMP-2,[8,10–14] or was only temporary ($uPA^{-/-}$, $Plg^{-/-}$).[7,9] Binding to specific cell surface receptors facilitates local activation of plasminogen at the cell surface[15] and restricts the activity of plasminogen activators and plasmin to the direct pericellular environment. Inhibition of plasmin activity to prevent SMC migration should occur directly at the cell surface to be most effective. However, the accessibility of receptor-bound plasmin to its natural inhibitors, such as α_2-antiplasmin and α_2-macroglobulin, is very low.[16]

This study describes the use of an adenoviral vector encoding a newly constructed hybrid protein designed to

Received June 26, 2000; revision received August 30, 2000; accepted August 31, 2000.
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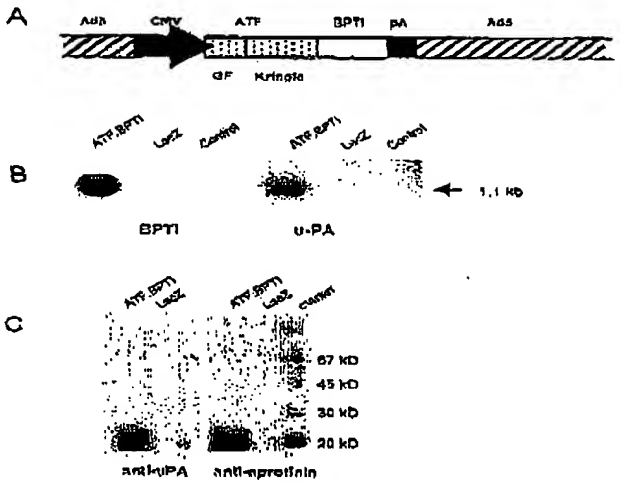


Figure 1. A, Structure of ATF.BPTI adenoviral vector. ATF.BPTI encoding DNA fragment consisting of ATF of uPA, containing growth factor domain (GF) and Kringle domain, and BPTI coding sequence is cloned into a E1-deleted adenoviral vector. Transcription is under control of a CMV promoter. B, CHO cells were infected with Ad.CMV.ATF.BPTI or Ad.CMV.LacZ (5×10^8 pfu/mL). After 48 hours, RNA was isolated. Northern blot analysis was performed with uPA and BPTI probes. Both probes hybridized with an mRNA of ≈1100 nucleotides, expected size for ATF.BPTI RNA in Ad.CMV.ATF.BPTI-infected cells. In Ad.CMV.LacZ-infected and noninfected cells, no signal was detected. C, Culture medium of Ad.CMV.ATF.BPTI- or Ad.CMV.LacZ-infected CHO cells was collected after 48 hours. Western blot analysis was performed with antibodies against uPA and aprotinin. Only in Ad.CMV.ATF.BPTI-infected cells did both antibodies detect a protein of ≈20 kDa.

inhibit plasmin activity directly at the cell surface as a new approach to preventing neointima formation. This hybrid protein consists of the amino-terminal fragment (ATF) of human urokinase plasminogen activator (uPA), which binds to the uPA receptor, linked to bovine pancreas trypsin inhibitor (BPTI), also known as aprotinin, a very potent inhibitor of plasmin. For application within the murine system, a variant of ATF.BPTI was constructed, mATF.BPTI, in which amino acid residues 22, 27, 29, and 30 of human uPA are replaced by their murine counterparts,[11] which can bind to the mouse uPA receptor (uPAR).

The effect of adenovirus-mediated expression of ATF.BPTI on neointima formation was studied in organ cultures of human saphenous vein. Furthermore, to test the efficacy of ATF.BPTI gene transfer in vivo, neointima formation was induced in the mouse femoral arteries as described by Moroi et al,[18] and mice were infected with Ad.CMV.mATF.BPTI.

Methods

Construction of ATF.BPTI Expression Plasmid and Adenoviral Vectors

pCMV.ATF.BPTI, an adenoviral shuttle vector encoding the ATF.BPTI hybrid protein, was constructed by deleting the DNA sequences encoding amino acids 139 to 401 from a uPA cDNA–containing plasmid,[17] resulting in a plasmid encoding the ATF and the 11 C-terminal amino acid residues of uPA, including the stop codon. Subsequently, a DNA fragment encoding amino acid residues 36 to 93 of BPTI, isolated by polymerase chain reaction (PCR) on genomic DNA from bovine aortic endothelial cells, was cloned into this vector. The ATF.BPTI-encoding DNA fragment was cloned in the adenoviral shuttle vector pCMV. For the generation of the recombinant adenovirus, pCMV.ATF.BPTI and pJM17 were cotransfected in HER9.11 cells[19] by standard procedures. Ad.CMV.mATF.BPTI was constructed by an identical procedure, with the (Tyr22, Arg27, 29, 30) uPA mutant cDNA[17] used as starting material. To construct Ad.CMV.BPTI, pCMV.BPTI was constructed by deleting the sequences encoding amino acids 2 to 137 from pCMV.ATF.BPTI by use of PCR. PCMV.ATF and pCMV.mATF were used to construct Ad.CMV.ATF and Ad.CMV.mATF. An "empty" control adenoviral vector (Ad.Control) was constructed by use of the pCMV vector without insert. For all adenoviral preparations, the particle-to-pfu ratios were determined and shown to be between 15 and 20.

An ATF.BPTI ELISA was set up with antibodies against ATF[20] as a catching antibody and against aprotinin (gift from Dr Emeis, Leiden, Netherlands) as a detecting antibody. With this ELISA, both ATF.BPTI and mATF.BPTI can be detected. For quantification, a standard of ATF.BPTI was prepared by infection of Chinese hamster ovary (CHO) cells, of which the ATF concentration was determined with a uPA ELISA.[20]

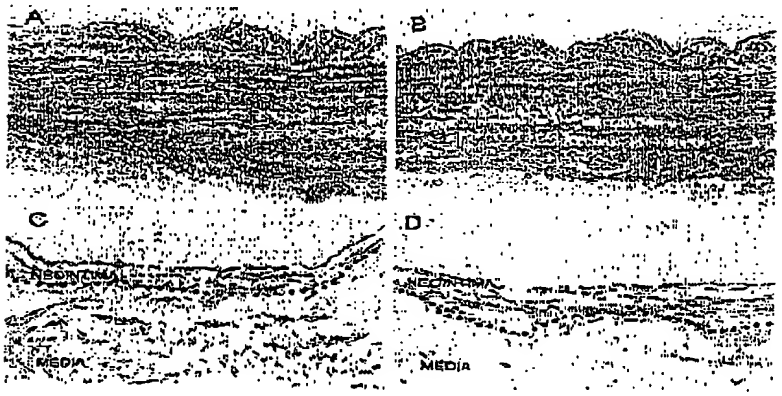


Figure 2. Human saphenous vein cross sections are stained for SMC α-actin (A, C) and uPAR (B, D). In sections of uncultured vessels (A, B), no neointima is present above SMC layer. In segments cultured 5 weeks (C through F), a loose neointimal structure is detectable, consisting primarily of SMC α-actin–positive cells (C). These cells also express uPAR (D).

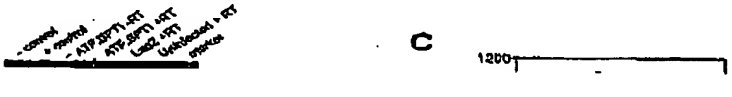
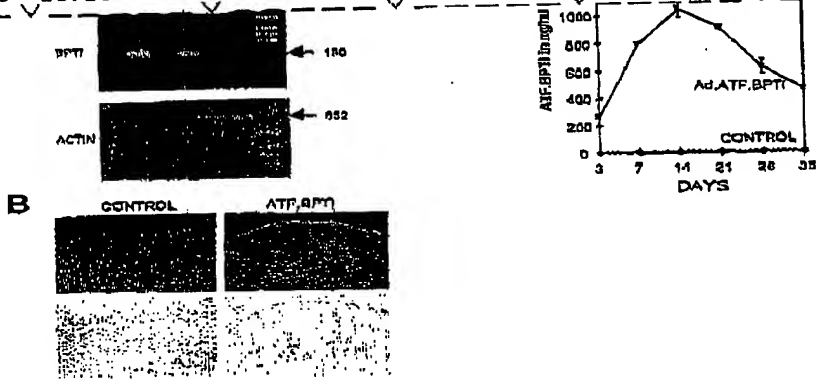


Figure 3. Human saphenous vein cultures were infected with 5×10^8 pfu/mL Ad.CMV.ATF.BPTI and analyzed for ATF.BPTI expression after 3 days. RT-PCR



analysis (A) was performed with BPTI-specific and actin-specific oligonucleotides; negative controls were H_2O (– control), an Ad.CMV.LacZ-infected, and an uninfected segment of same vessel, as well as incubation without RT; pCMV.ATF.BPTI was a positive control (+ control). Sections (5 μm) of uninfected and AdCMV.ATF.BPTI-infected segments were used for in situ hybridization (B) with a ^{35}S-labeled uPA probe. Both endogenous uPA and induced ATF.BPTI mRNA expression can be detected. ATF.BPTI mRNA expression is detectable at luminal site of infected segments. Lower panels are bright-field illuminations of upper panels. C, Conditioned medium of Ad.CMV.ATF.BPTI-infected and uninfected control cultures were collected at various time points, and ATF.BPTI expression was measured by ELISA. ATF.BPTI levels are expressed in ng/mL (mean±SEM, n=3).

Plasmin activity was analyzed by use of the chromogenic substrate S2251 (Chromogenix). Diluted samples of the conditioned CHO medium (2 μg/mL ATF.BPTI, or 0.1 μmol/L) were incubated for 15 minutes with 500 pmol/L plasmin. Then S2251 was added, and after a 24-hour incubation at 37°C, the A_{405} was measured. As a control, plasmin was incubated with buffer or with 10 or 100 pmol/L aprotinin (Trasylol, Bayer). For detection of plasmin activity in vascular tissue, extracts of 4 murine aortas were prepared as previously described[21] and pooled. After 15 minutes of incubation at room temperature with 100 pmol/L plasmin, S2251 was added, and the activity was measured in the absence and presence of aprotinin-inhibiting antibodies to discriminate between intrinsic α_2-antiplasmin activity[22] and BPTI-mediated plasmin inhibition.

Saphenous Vein Organ Cultures and Analysis of Neointima Formation

Segments of saphenous vein were obtained from patients undergoing CABG surgery, according to the guidelines of the Review Board of the Leiden University Medical Center. The veins were cultured as described previously.[23,24] From every patient, 1 segment was infected for 1 hour with 5×10^9 pfu Ad.CMV.ATF.BPTI in 1 mL at 37°C with gentle shaking, and 1 was mock-transfected. From 3 patients, segments were infected with 5×10^9 pfu Ad.CMV.ATF, Ad.CMV.BPTI, or Ad.Control or mock-transfected. Vein segments were cultured for 5 weeks and analyzed histochemically. The viability of cultures during 5 weeks was demonstrated by an unchanged ATP production during the culture period (not shown). Neointima formation in the treated segments was always compared with their untreated counterparts and quantified on multiple sections (n=6) of the segments with the QWin image analysis system (Leica).

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded sections with monoclonal antibodies against α-SM-actin (Sigma), uPA receptor (H2, gift from Dr Weidle, Penzberg, Germany), von

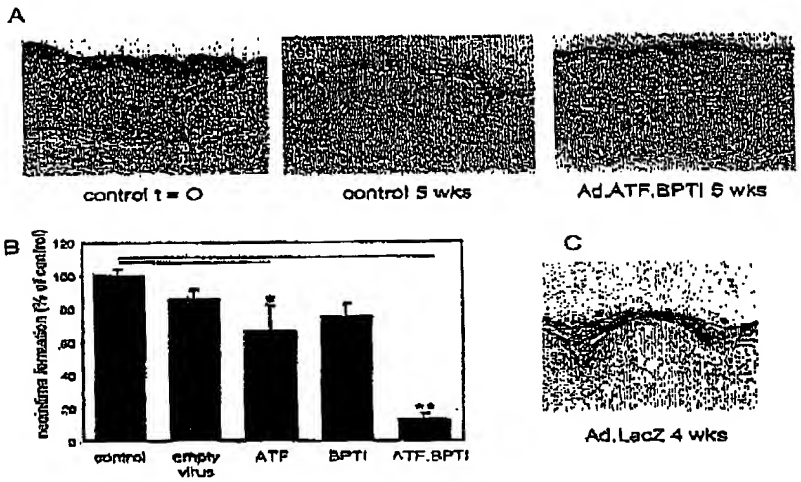


Figure 4. A, Segments of human saphenous vein were infected with Ad.CMV.ATF.BPTI (1 hour, 5×10^9 pfu/mL) and cultured 5 weeks. Uninfected counterparts of same patient were used as controls. Representative histological sections of cultures are shown. After 5 weeks in control sections, a multilayer neointima was observed, whereas in Ad.CMV.ATF.BPTI-transduced sections, virtually no neointima was present, similar to uncultured control. B, Neointima size in cultured segments was quantified in multiple sections (n=6) per segment, pairwise in treated and untreated counterparts for Ad.CMV.ATF.BPTI (n=10). From 3 patients, segments were infected with Ad.CMV.ATF, Ad.CMV.BPTI, Ad.Control, or mock-transfected (percentage of control, mean±SEM, *P<0.05, **P<0.001). C, Cultures were infected with 5×10^9 pfu/mL Ad.CMV.LacZ and analyzed for β-galactosidase activity after 28 days. Newly formed neointima consisted of intensely blue-staining cells.

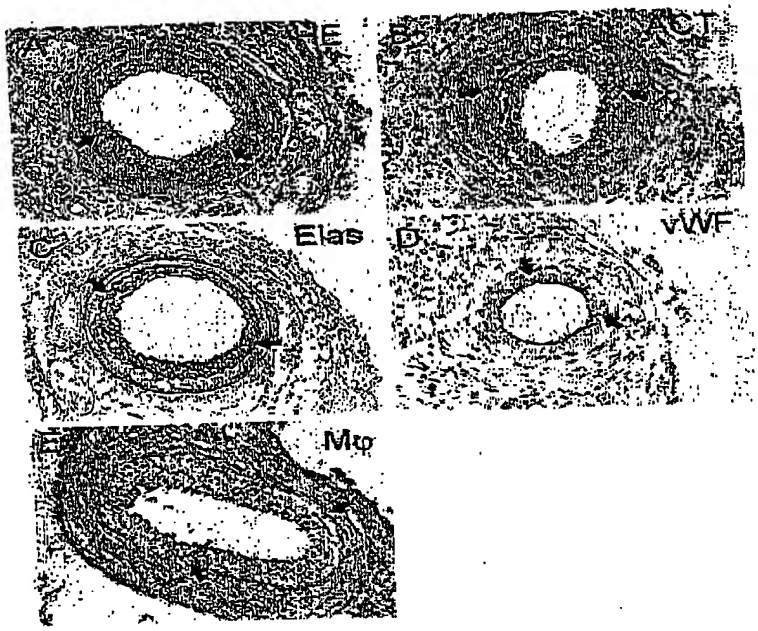


Figure 6. Neointima formation in murine femoral arteries was characterized 3 weeks after cuff placement. Arrows indicate internal elastin lamina. A neointima of 4 to 6 cell layers was detected (A, hematoxylin [HE] staining), consisting primarily of SMC α-actin–positive cells (ACT, B), on top of an internal elastic lamina, indicated by arrows (C, Weigert's elastin staining [Elas]), beneath an apparently intact endothelial layer (D, von Willebrand factor [vWF] staining). Macrophages (Mφ) were detected only in granulation tissue surrounding vessel within cuff, but not in neointima (E).

Willebrand factor (DAKO A/S), the macrophage-specific monoclonal antibody AIA31240 (Accurate Chemical), and polyclonal antibodies against uPA[24] and aprotinin.

Bound antibodies were detected with horseradish peroxidase–conjugated rabbit anti-mouse antibodies or swine anti-rabbit antibodies (Dako A/S). Sections were counterstained with hematoxylin. In situ hybridization was performed as described[25] with 0.7-kB ^{35}S-labeled riboprobes for human uPA.

Femoral Artery Cuff Placement

All animal experiments were approved by the Animal Welfare Committee of TNO. C57BL/6 mice (18 to 25 g) were anesthetized with Hypnorm (Bayer) and Dormicum (Roche) (25 mg/kg each). The left femoral artery was isolated from surrounding tissues, loosely sheathed with a 1.0-mm polyethylene cuff (PE-50 tubing; ID, 0.4 mm; OD, 0.8 mm; Becton Dickinson) as described previously.[16] The right femoral vein was dissected and used for intravenous injection with viral vectors (2×10^9 pfu in 200 μL). Animals were euthanized after 19 days. After perfusion fixation, tissue segments were embedded in paraffin. Neointima formation was quantified by image analysis in 6 representative serial sections per vessel segment.

Statistical Analysis

Data are presented as mean±SEM. Statistical analysis of neointima formation in organ cultures was performed with 1-way ANOVA followed by Fisher's least significant difference test. For the in vivo experiments, overall comparisons between groups were performed with the Kruskal-Wallis test. If a significant difference was found, groups were compared with their control by Mann-Whitney rank sum tests. Probability values of $P<0.05$ were regarded as significant.

Results

In Vitro Characterization of ATF-BPTI

An adenoviral vector (Ad.CMV.ATF.BPTI) was constructed that was capable of directing the expression and secretion of the hybrid protein ATF.BPTI, consisting of ATF and BPTI (Figure 1A). In parallel, Ad.CMV-mATF.BPTI, encoding the murine residues Tyr22, Arg27, Arg29, and Arg30 in the human ATF domain, was constructed. Northern blot analysis of Ad.CMV.ATF.BPTI-infected CHO cells revealed a hybridization signal for both uPA and BPTI cDNA probes with an RNA of ≈1100 nucleotides, the expected size of ATF.BPTI mRNA (Figure 1B). By Western blot analysis of conditioned medium of Ad.CMV.ATF.BPTI-infected CHO cells with antibodies against human uPA to detect the ATF domain and antibodies against aprotinin to detect the BPTI domain, the presence of the 2 domains in ATF.BPTI was confirmed (Figure 1C). ATF.BPTI protein production in Ad.CMV.ATF.BPTI-infected CHO cells was also demonstrated by ELISA (data not shown).

Inhibition of plasmin activity at the surface of human saphenous vein SMCs was achieved by incubating SMCs for 1 hour with ATF.BPTI-containing CHO cell culture medium. After extensive washing, extracts were prepared and the plasmin inhibitory capacity was determined. Plasmin activity was inhibited by 85.2±3.9% with a 1:4 dilution of CHO medium. Incubation with increasing concentrations of a uPAR-blocking antibody (H2) reduced plasmin inhibition dose-dependently (not shown). Incubation with anti-aprotinin antibodies resulted in a total reduction of the plasmin inhibition. No plasmin inhibitory activity could be detected in lysates of cells that underwent a mild acid treatment (2 minutes in pH 3.0 glycine buffer), demonstrating that the inhibitory activity can be removed

from the uPAR. Similarly, 94.7±1.5% inhibition of plasmin activity at the surface of murine endothelioma cells by mATF.BPTI was demonstrated. These results indicate that ATF.BPTI binds to the uPAR and can inhibit plasmin activity at the cell surface.

Effect of Ad.CMV.ATF.BPTI Infection on Neointima Formation in Human Saphenous Vein Organ Cultures

In human saphenous vein organ cultures, a multilayer neointima is formed in 5 weeks that consists mainly of α-SMC actin–positive cells, either SMCs or myofibroblasts. These cells originate from the media and adventitia (Figure 2A and 2C). uPAR expression was detected in cells of the media (also before culture [Figure 2B]) and neointima (Figure 2D), as well as in the adventitia. Staining for uPAR in media and neointima indicates that the receptor, to which ATF.BPTI is expected to bind, is present in the target cells in the saphenous vein.

Efficient transduction of saphenous vein segments with Ad.CMV.ATF.BPTI and subsequent expression of ATF.BPTI were monitored by reverse transcription (RT)-PCR, in situ hybridization, and ELISA (Figure 3).

To assess whether adenovirus-mediated overexpression of ATF.BPTI in saphenous vein segments would inhibit neointima formation, segments were infected with Ad.CMV.ATF.BPTI and cultured for 5 weeks. A nearly complete reduction in neointima size was observed in all the sections studied. Representative histological sections are shown in Figure 4A. In the control section, after 5 weeks, a multilayer neointima can be observed, whereas in the Ad.CMV.ATF.BPTI-transduced section of the same patient, virtually no neointima is present, comparable to a segment harvested at the start of the culture (Figure 4A). Neointima formation was quantified by image analysis in multiple sections of the segments, pairwise in treated and untreated counterparts, and the rate of inhibition was calculated. The mean neointimal area was 11.2±1.6 mm^2 (no virus) compared with 1.2±0.3 mm^2 (Ad.CMV.ATF.BPTI virus). The mean inhibition of neointima formation is 87.5±3.8% (mean±SEM, n=10, $P<0.001$) (Figure 4B). After transduction with 5×10^9 pfu Ad.CMV.Control, Ad.CMV.ATF, or Ad.CMV.BPTI, the inhibition of neointima formation was not significantly different from the mock-transfected control segments, except for Ad.CMV.mATF ($P=0.018$). After transduction with 5×10^9 pfu AD.CMV.LacZ, intense blue staining of the neointima can be observed (Figure 4C), indicating that efficient transduction of the segments can be obtained.

Effect of Ad.CMV.mATF.BPTI Infection on Neointima Formation In Vivo

Neointima formation was induced in vivo in murine femoral arteries by placement of a 0.4-mm polyethylene cuff. Cuff placement resulted in profound neointima formation in 3 weeks, consisting of ≈4 to 6 layers of SMCs, as described previously[18] (Figure 5A). The cuff-induced neointima consisted primarily of SMCs (Figure 5B) on top of the internal elastic lamina (Figure 5C), beneath a layer of von Willebrand factor–positive endothelial cells (Figure 5D). Macrophage infiltrates were not detected in the neointimal area but were present in the granulation tissue within the cuff, surrounding the vessel (Figure 5E).

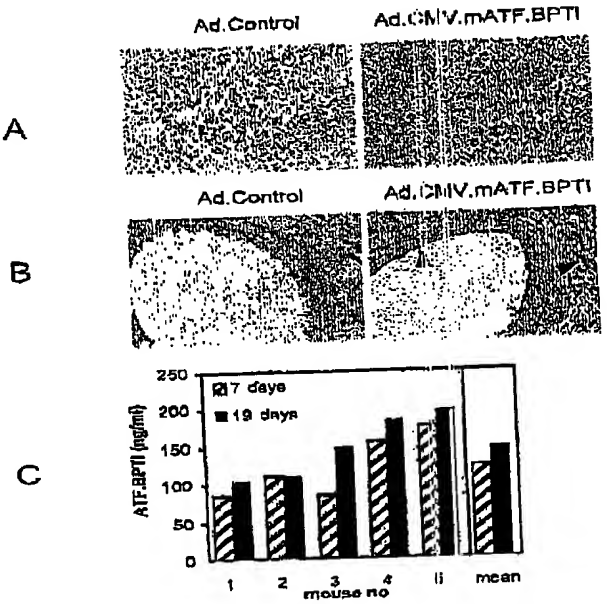


Figure 6. Mice were infected with Ad.CMV.mATF.BPTI (10^9 pfu in 200 μL) intravenously in femoral vein. Liver cross sections (A) of mice killed 5 days after infection were analyzed for ATF.BPTI expression. Positive cells, staining black, were observed in Ad.ATF.BPTI-infected and not in empty virus (Ad.Control)–infected mice. In cuffed arteries (B), positive cells, staining black, were detectable in intima and adventitia (arrow) after Ad.CMV.ATF.BPTI infection, and not after Ad.Control infection. In 5 mice, ATF.BPTI plasma levels were analyzed 7 and 19 days after infection by ELISA (C).

Mice were infected with Ad.CMV.mATF.BPTI (10^9 pfu in 200 μL) in the femoral vein and were killed 5 days later. ATF.BPTI was detected by immunohistochemistry with anti-aprotinin antibodies in liver parenchymal cells (Figure 6A) and in the cuffed arteries of Ad.CMV.mATF.BPTI-infected mice. The most prominent staining was near the luminal side, but positive cells could also be detected in the deeper layers of the vessel wall (Figure 6B). After 5 days, plasma levels of 40 ng/mL ATF.BPTI were reached. In aorta tissue extracts, 1 ng/mg ATF.BPTI was present. Furthermore, in the vessel wall, extracts of Ad.CMV.mATF.BPTI-infected mice, in addition to the ubiquitously present α_2-antiplasmin activity[22] (able to inhibit 65.3±3.5 μ-casein units (CU) plasmin [mean±SEM, n=3]), an aprotinin-specific plasmin inhibitory activity was detected, able to inhibit 11.2±2.9 μCU (equivalent to 0.45 ng active plasmin) per milligram of tissue extract. This inhibitory activity was blocked by antibodies against aprotinin. In extracts of control mice, only α_2-antiplasmin activity, and no BPTI-mediated plasmin inhibition, could be detected.

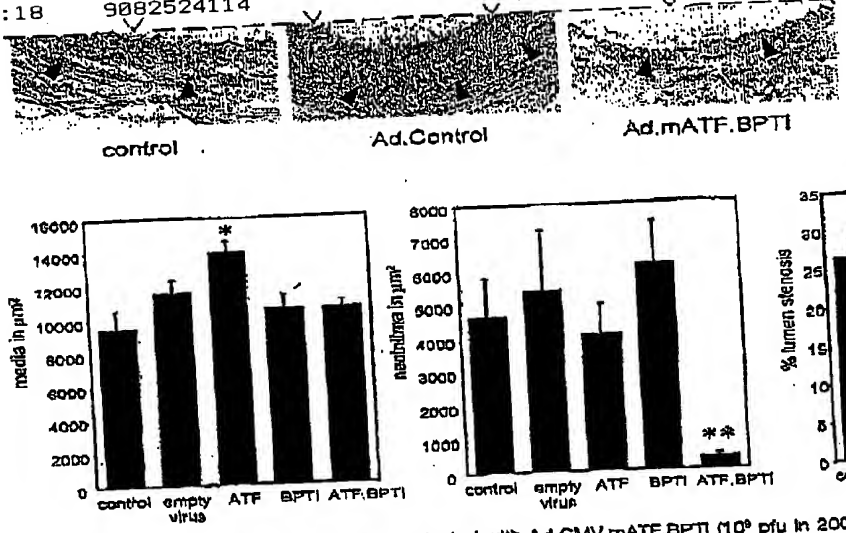


Figure 7. Directly after cuff placement, mice were infected with Ad.CMV.mATF.BPTI (10^9 pfu in 200 μL) intravenously. Mice were killed 19 days later, and neointima formation was analyzed. Mock-infected and Ad.Control-infected mice were used as controls. Representative histological sections are shown (A). Arrowheads indicate internal elastic lamina. B, Media (in μm^2), neointima, and percentage of luminal stenosis in cuffed arteries were quantified in multiple cross sections of segments, in mock-infected, Ad.Control-, Ad.CMV.mATF.BPTI-, Ad.CMV.mATF-, and Ad.CMV.BPTI-infected mice (mean±SEM, n=6, *P<0.5, **P<0.001).

Next, mice were infected with Ad.CMV.mATF.BPTI, Ad.CMV.mATF, Ad.CMV.BPTI, or Ad.Control in the contralateral femoral vein. ATF.BPTI expression was monitored by ELISA (Figure 6C), and 19 days after infection, Ad.CMV.mATF.BPTI plasma levels of 136±19 ng/mL ATF.BPTI, 118±11 ng/mL ATF, and 65±13 ng/mL BPTI were detectable. Mice were killed on day 19, and cuffed vessel segments were analyzed for neointima formation. In Ad.CMV.mATF.BPTI-treated animals, the neointima is maximally 1 or 2 cell layers thick, whereas in untreated control mice, Ad.Control- (Figure 7), Ad.CMV.mATF-, or Ad.CMV.BPTI-infected mice (not shown), the neointima is ~4 to 6 cell layers thick. Quantification revealed no significant difference in neointima formation in either mock-infected, Ad.Control-, Ad.CMV.mATF-, or Ad.CMV.BPTI-infected mice, whereas in Ad.CMV.mATF.BPTI-infected mice, a dose-dependent reduction of neointima formation can be observed (Figure 7B), with a mean neointima area of 333±127 μm^2 (mean±SEM, n=6) after infection with 10^9 pfu. No decrease in media size could be observed, only a small but significant increase in media area in the Ad.CMV.mATF-infected mice. The mean percentage of luminal stenosis was reduced from ~25% to 2% in the Ad.CMV.mATF.BPTI-infected mice.

Discussion

In this report, adenoviral gene transfer of a novel hybrid protein, ATF.BPTI, was analyzed in 2 independent models for neointima formation, a human in vitro model for vein graft neointima formation and a murine in vivo model for neointima formation. It was demonstrated that this secreted, cell surface–binding serine protease inhibitor strongly reduces SMC migration and neointima formation in both human and murine blood vessels. Whereas the α2-antiplasmin activity ubiquitously present in the vessel wall seems to have no effect on neointima formation, the cell surface–targeted ATF.BPTI does so very efficiently. ATF and BPTI had no significant effect on neointima formation. This indicates that cell surface targeting of BPTI largely improves the efficacy of plasmin inhibition in preventing neointima formation.

Several gene transfer approaches have been used to inhibit intimal hyperplasia and SMC accumulation. Most of these studies are directed at inhibiting SMC proliferation after vessel wall injury. Recently, several studies reported partial or temporal inhibition of neointima formation by use of protease inhibitors, TIMP, or PAI-1.[8,10] The genes used thus far, such as HSV-tk, p21, hirudin, TIMP1, and PAI-1,[1,3,8,10,27] are all existing genes, in contrast to the new recombinant gene we describe here.

In this study, we first focused on inhibition of SMC migration in human vein graft neointima formation. Saphenous vein organ cultures were used as a human model for